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Ectomycorrhizae between *Amanita rubescens* and Himalayan spruce (*Picea smithiana)* from Pakistan

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Abstract — Field ectomycorrhizae of *Amanita rubescens* with *Picea smithiana* are described for the first time. Ectomycorrhizal roots were sampled beneath the sporocarp from the rhizosphere of the host plant. The sporocarps and ectomycorrhizae were characterized morphologically and anatomically. The most important characters of the ectomycorrhizae are a monopodial mycorrhizal system, white to cream colour of the mantle, hyaline emanating hyphae, and thick hairy rhizomorphs emerging mostly from restricted points. All mantle layers are plectenchymatous. Hyphae lack clamp connections forming rings. Rhizomorphs are composed of thick central hyphae without septa. The diameter of the rhizomorphs is narrower than other known ECM of *Amanita* spp. This association is reported for the first time from Pakistan.

Key words — *Amanitaceae*, ectomycorrhizogenous, Mukshpuri, *Pinaceae*

Introduction

Himalayan spruce (*Picea smithiana* (Wall.) Boiss.) is native to western Himalaya and adjacent mountains from northeast Afghanistan to central Nepal. It grows at altitudes of 2400–3600 m a.s.l. in forests together with *Cedrus deodara* (Roxb.) D. Don, *Pinus wallichiana* A.B. Jacks., and *Abies pindrow* (Royle ex D. Don) Royle. It is valued for its use in the pulp industry as a source of paper. Although it is of marginal use as fuel, it yields a fairly good charcoal (Hill 1952). In Pakistan Himalayan spruce is found in northern Pakistan (Kurram Agency, Chitral, Swat and Gilgit eastwards), Murree hills (Nathia Gali, north slope of Mukshpuri) and Kashmir (Stewart 1972). This tree grows in acidic, neutral and basic (alkaline) soils as well as in very acidic and nutritionally poor soil. *Picea smithiana* overcomes all these extreme soil conditions due to its ability to form ectomycorrhizae with forest mycoflora (Huxley 1992). Field ectomycorrhizae (ECM) of *Picea* have already been described. About 162 ECM morphotypes of different macrofungi have been recorded with *Picea* spp. (De Roman et al.

2005, Agerer 2008), but none with species of *Amanita*. *Amanita* spp. have been reported as ectomycorrhizal with *Betula pendula, Cedrus deodara* and *Pinus* spp., (Cuvelier 1990, Daniele et al. 2005, Agerer 2006).

Amanita is one of the most studied and well-known agaric genera. It is characterized mainly by its terrestrial, mostly ectomycorrhizogenous and fleshy basidiomes (Gonzalez et al. 2002). In Pakistan, *Amanita* is represented by 12 species, mostly in Himalayan moist temperate forests. These forests are a hot spot for the diversity of ectomycorrhizal macromycetes particularly amanitas from this country (Tulloss et al. unpublished data). *Amanita rubescens* Pers. has been reported from Murree Hills and northern areas of Pakistan under *Abies pindrow*, *Picea smithiana* and *Pinus* spp. in coniferous forests (Murakami 1993). This fungus was originally described in northern Europe associated with *Quercus* and *Fagus.* Neville & Poumarat (2004) mention its association with *Abies alba* Mill., *Pinus pinaster* Aiton, *P. sylvestris* L. as well as *Castanea sativa* Mill., *Quercus ilex* L., *Q. pubescens* Willd., and *Q. suber* L.

During the study of biodiversity of mushrooms and ectomycorrhizae of Himalayan moist temperate forests of Pakistan (Niazi et al. 2006, 2007), *Amanita rubescens* was found under *Picea smithiana*, and their association was confirmed by tracing the mycelial connection from fruit body to the morphotypes (Agerer 1991).

The morphological and anatomical description of mycorrhizae (Miller et al. 1991) and identification of their fungal partners are pre-requisites for recognizing mycorrhizal diversity in an ecosystem. In the present study, field ectomycorrhizae of *A. rubescens* and *P. smithiana* are reported and characterized in detail for the first time from Pakistan.

Materials and methods

Study Area

The study area is located in Murree Hills of Himalayan moist temperate forests of Pakistan. These forests present dominant stands of *Abies pindrow* with varying stands of *Cedrus deodara, Picea smithiana, Pinus wallichiana*, and *Taxus wallichiana* Zucc. intermixed with deciduous or broad leaved trees. The dominant understory vegetation consists of patches of *Viburnum cotinifolium* D. Don, *Skimmia laureola* (DC.) Dcne., and *Urtica dioica* L., among others (Stewart 1972). All these sites are located between 1830 and 3050 m a.s.l. with an annual precipitation between 5000 and 6000 mm (Champion et al. 1965).

Sampling and identification

The study area was visited during rainy seasons from July, 2006 July, 2007. Sporocarps were photographed and field notes were taken. Soil blocks of 10 × 5 cm were concurrently collected below fruit bodies. The rooted soil samples were taken out with a sharp digger and then wrapped in polythene bags to

avoid evaporation. Samples were examined under a stereomicroscope at 40× magnification for hyphal connections leading from sporocarps to fungal mantle (Agerer 1991). ECM morphotypes were placed in water and cleaned with a camel hair brush. These were photographed under a Wild Heerbrugg stereo microscope.

Microscopic characterization

The epigeous basidiocarps were analyzed macroscopically (colour, lamellae, shape.) and microscopically (basidia, basidiospores, cystidia) following Reid's (1984) methodology. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, (LAH) Pakistan. Morphological and anatomical characterizations of the mycorrhizae were carried out by following the terminology of Agerer (1991). Individual tips of mycorrhizae were examined under the microscope. These surface views were observed in detail; measurements were taken and traced out using a camera Lucida. The internal structures of rhizomorphs, and emanating hyphae were also measured and traced out. Color Reactions of fungal tissues of morphotypes were noted with Melzer's reagent, 10% KOH, and lactic acid.

Results

The direct attempt to identify ectomycorrhizae of *Amanita rubescens* by tracing hyphal connections between the stipe base of sporocarp and mycorrhizal root tips was successful.

Description of the basidiocarp of *Amanita rubescens* (Fig. 1 A)

Pileus 60–105 mm wide, reddish brown at centre, white to cream at margin, hemispheric at first, then convex to broadly convex to planar, umbonate, shiny, dry, non appendiculate and non striated margins: Flesh up to 4–5.5 mm thick at disk, Universal veil present, prominent as oblong to roundish dull red or reddish brown to pale brownish gray small warts, easily removable. context white, turning reddish when bruised or cut. margins without striation or minutely striated. Lamellae free to adnate, crowded, white, sometimed discoloring reddish, 5–15 mm broad. lamellulae truncate, common, evenly distributed, of diverse length, present between every pair of lamellae. Stipe 50–145 × 10–23 mm, equal or slightly bulbous toward base, notably longer than pileus width; base with volval scales or zones, without rim: whitish becoming stained pinkish to dirty red, smooth but sometimes with flakes. partial veil superior, thick, membranous, moderately broad, skirt-like, persistent, becoming cream, collapsing and tearing, up to 12 mm long. universal veil present as white or pinkish flakes or warts on lower side of stipe. Odor and taste not recorded.

Macrochemical Tests: none performed.

Basidiospores: (7.5–)8.0–10.5(–12.5) × (5.0–)5.4–7.0(–8.0), ellipsoid to broadly ellipsoid or elongated, hyaline, thin-walled, smooth, amyloid, subglobose to ellipsoid; apiculus sublateral, cylindric; contents dominantly monoguttulate, with or without additional small granules, occasionally granular; color in deposit white. Basidia: 18–24 × 8–10 µm, 2–4 sterigmata, thin walled, clavate.

Habitat And Distribution: Solitary, under *Picea smithiana*. Pakistan: At 2400–2580 m a.s.l.

Collections Examined: PAKISTAN: N.W. FRONTIER PROV.—Hazara Distr. – On the way towards Mukshpuri, 12.viii.2006 A. R. Niazi 12806 (LAH).

Description of ECM of *Amanita rubescens*

Morphological characteristics (Fig. 1 B)

Mycorrhizal system monopodial pinnate, 4–12 mm long with axis 0.5–2 mm thick, sometime profusely branched forming clusters, white when young, turns pale brown when old. Thick mantle layer appearing silvery, opaque, no host tissue visible, but in few places white patches. Unramified ends straight to slightly bent, (0.5–)1.5–2.5(–5) mm long, 0.4–0.6 mm diam, slightly swollen. Mantle surface white to shiny, smooth to rough due to soil particles, water and air trapped among mantle hyphae. Emanating hyphae frequent, white, and thin, up to 3 µm, sometimes surrounding the unramified ends. Rhizomorphs abundant, white, up to 0.15 mm thick, roundish in cross-section, smooth or hairy with few emanating hyphae, rhizomorphs frequently and repeatedly branched, slightly swollen at base, arising mostly from restricted points, rarely from base. Sclerotia lacking.

Anatomical characteristics of mantle in plan views (Fig. 1 C–D)

Mantle plectenchymatous in all layers. Outer Mantle layer plectenchymatous, loosely net-like arrangement, hyphae branched and septate; rounded and cylindric hyphae, 14–25 µm long, 2–3.5 µm diam, cell wall thick, up to 2 µm; sometimes in ring-like arrangement, simple H-type anastomosis among few parallel hyphae, white to hyaline, smooth surface, without clamps. Inner Mantle layer densely plectenchymatous; hyphae closely packed with few interhyphal spaces, entangled with each other, branched, septa without clamps; hyphal cells rounded to cylindric, hyaline , (10–)14–17(–24) µm long, 2–3 (–4) µm diam, smooth-walled, simple or fused anastomoses;. Very tip slightly yellowish, plectenchymatous arrangement, hyphae forming a dense mycelial mat, 3–4.5 µm diam. CROSS Section mantle 42 µm thick, differentiated into plectenchymatous layers, cortical cells with Hartig net in 2–3 rows.

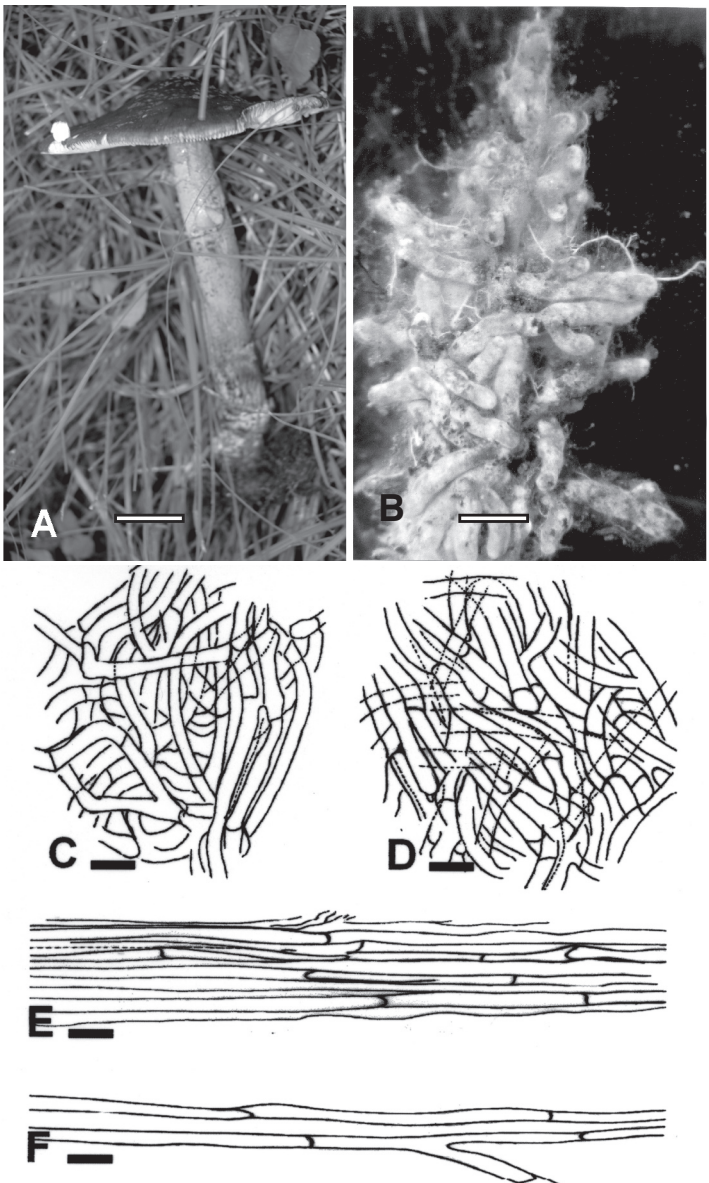

Fig. 1. (**A**) Amanita rubescens (**B**) Ectomycorrhizal system (**C**) outer mantle layer (**D**) inner mantle layer (**E**) Internal view of rhizomorph (**F**) Emanating hyphae. Scale bar = 2 mm for A and B, 20 *µm for* C–F.

Emanating hyphae emerging out from outer surface. Longitudinal Section plectenchymatous hyphae running parallel, entangled with each other forming a compact inner layer, penetrating into cortical region, forming dense finger-like network among cortical cells, reaching to endodermis.

Anatomical characteristics of emanating elements (Fig. 1 E–F)

Rhizomorphs up to 130 µm thick, wide, rounded to flat, composed of closely packed parallel hyphae; hyphae smooth walled, rarely branched and septate; hyphal cells rounded to cylindric, 10–16 µm long, 3–4 µm diam, cell walls thick, up to 0.5 µm; sometime fused with each other with or without any particular type of anastomosis. A wide central hypha also observed, up to 5 µm diam, thick walled, up to 0.5 µm, incomplete septa without clamp connections. Emanating hyphae white, 2–3 µm diam, 150 µm long, straight, septate, without clamps, branched. Cystidia and chlamydospores not observed.

Color Reactions: 10% KOH-no reaction, Melzer's Reagent-bluish, Lactic acid-no reaction.

Voucher Specimen: Ectomycorrhizae under *Picea smithiana*, in Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan. A. R. Niazi #AR1021 (LAH). Fruit bodies examined; Pakistan, Khanspur-Ayubia, at 2135 m (LAH).

Discussion

The *Amanitaceae* is a well known ectomycorrhizal family that falls in the Euagaric clade (Binder et al. 2005). Up to now, field mycorrhizae of three *Amanita* spp. have been described in detail: *A. citrina* Pers. (Mleczko 2004), *A. muscaria* (L.) Lam. (Uhl 1988) both with *Pinus sylvestris* and *Cedrus deodara* (Daniele et al. 2005), *A. strobiliformis* (Paulet ex Vittad.) Bertill. with *Betula pendula* Roth (Raidl & Verma 2006, Agerer 2006). The ectomycorrhizal fungus, *Amanita rubescens*, is reported here for the first time growing under *Picea smithiana* and associated mutualistically, and their mycorrhizae are morphologically and anatomically described. The ECM morphotypes of these *Amanita* spp. including the one described here share common features such as a smooth to silvery mantle surface of white to off-white to yellowish colour, plectechymatous outer mantle, and the presence of rhizomorphs with a wider central hyphae and the ring-like arrangement of hyphae in the outer plectenchymatous layer.

The ECM similarities between *A. rubescens–P. smithiana* and *A. strobiliformis –B. pendula* are a plectenchymatous mantle in all layers and a compact rhizomorph with few emanating hyphae. However, *A. strobiliformis* ECM can be separated from the ECM of *A. rubescens* since it presents geniculate hyphae in the mantle surface, a wider central rhizomorph hypha, and a negative Melzer's reaction.

All known *Amanita* ECM morphotypes can also be differentiated based on the size of the central hyphae of their rhizomorphs. The rhizomorph of *A. rubescens* differs from the other three morphotypes by its much narrower central hyphae, up to 5 µm, compared with central hyphae up to 10 µm in *A. strobiliformis*, 15 µm in *A. muscaria*, and 24 µm in *A. citrina*. The rhizomorphs of *A. rubescens* do not contain crystals, while *A. citrina* has characteristic angular crystalloids in its rhizomophs (Mleczko 2004).

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